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Pleurotus eryngii species complex: Sequence analysis and phylogeny based on partial *EF1α* and *RPB2* genes

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ABSTRACT

The *Pleurotus eryngii* species complex comprises at least six varieties (var. *eryngii* (DC.: Fr) Quel., *ferulae* Lanzi, *elaeoselini* Venturella *et al.*, *nebrodensis* (Inzenga) Sacc., *tingitanus* Lewinsohn *et al.* and *tuoliensis* C.J. Mou). This species is unique among the genus *Pleurotus* because in nature it is found in association with certain species of the Apiaceae (Umbelliferae) and Asteraceae (Compositae) families. Sequences of partial regions of the translation elongation factor (*EF1α*) and RNA polymerase II (*RPB2*) genes were analyzed in order to detect nucleotide polymorphisms that might unequivocally distinguish varieties *eryngii*, *ferulae*, *elaeoselini* and *nebrodensis*. A phylogenetic analysis was also performed with an aim to establish phylogenetic relationships among those. Sequence analysis of the partial *EF1α* and *RPB2* genes contained nucleotide polymorphisms able to unequivocally distinguish variety *nebrodensis* from the rest. However, distinction among *eryngii*, *elaeoselini* and *ferulae* was achieved only through the *RPB2* gene. The phylogenetic analyses from the combined data sets (*EF1α* and *RPB2*) indicated that *P. eryngii* is a monophyletic group and that varieties *eryngii*, *elaeoselini* and *ferulae* are closely related. *P. eryngii* var. *nebrodensis* was placed in a distinct clade clearly differentiated from the other varieties but still monophyletic with the *P. eryngii* complex. The limited nucleotide variation in partial *EF1α* and *RPB2* among varieties *eryngii*, *ferulae* and *elaeoselini* supports the placement of these groups as varieties and not species within the complex.

Introduction

The genus *Pleurotus* includes several edible species that are known for their exceptional flavor and their relatively low-cost methods of cultivation. Species identification in this genus is often difficult because it is largely based on morphology of the basidiomata. Incorrect naming of commercial strains has also contributed to ambiguity in the taxonomy of the genus (Buchanan 1993). The "species complex" concept is widely applied in fungi to define closely related species that are completely or partially intercompatible (Vilgalys & Sun 1994; Zervakis & Balis 1996; Zervakis *et al.* 2001b; Bao *et al.* 2004). The *Pleurotus eryngii* species complex consists of several varieties and species: var. *eryngii* (DC.: Fr) Quel, var. *ferulae* Lanzi (syn = *Pleurotus fuscus* var. *ferulae*), var. *elaeoselini* Venturella *et al.*, var. *tingitanus* Lewinsohn *et al.*, var. *nebrodensis* (Inzenga) Sacc, var. *tuoliensis* C.J. Mou, *Pleurotus hadamardii* Costantin and *Pleurotus fossulatus* (Cooke) Sacc. (Candusso & Basso 1995; Venturella 2000; Lewinsohn *et al.* 2002; Kawai *et al.* 2008). In nature, members of this group function as

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facultative biotrophs associated with some genera of the Apiaceae (Umbelliferae) and Asteraceae (Compositae) families (Zervakis et al. 2001a). Morphological characteristics used to differentiate individuals within these groups may be ambiguous because of environmental influences or overlap of traits of interest. For example, it was reported recently that white basidiomata, a feature considered distinctive of var. *nebrodensis*, may also be found in some isolates of variety *ferulae* growing in association with *Ferula sinkiangensis* in Xinjiang, China (Zhang et al. 2006). As is true for other fungi, incomplete reproductive barriers exist within the *P. eryngii* group (Bresinsky et al. 1987, Zervakis & Balis 1996, Kawai et al. 2008). Therefore, biological species delimitations often are difficult to establish.

The authors previously evaluated the utility of the internal transcribed spacer region (ITS) of the rDNA gene cluster and partial β-tubulin gene to infer phylogenetic relationships among varieties of *P. eryngii* (Rodriguez Estrada 2008; Rodriguez Estrada et al. 2008). Allelic polymorphisms (intra-isolate polymorphisms) within the *β-tubulin* gene and lack of variation in the ITS region (also reported by Ro et al. 2007) were observed. Therefore, those regions were neither useful for comprehensive phylogenetic studies within this species complex nor to unequivocally distinguish varieties. Other protein-encoding regions that may be useful to determine phylogenetic relationships among closely related taxa are the genes encoding for the translation elongation factor (*EF1α*) and the second largest subunit of the RNA polymerase II (*RPB2*) (Liu et al. 1999; Roger et al. 1999; Matheny et al. 2002; Tanabe et al. 2004; Froslev et al. 2005; Matheny 2005; Matheny et al. 2006).

The translation elongation factor (*EF1α*) is a binding protein required for ribosomal protein synthesis in eukaryotes. A study performed by Marongiu et al. (2005) revealed that the *EF1α* gene contains nucleotide substitutions that are useful to distinguish two varieties of *P. eryngii*: *ferulae* and *eryngii*. The *RPB2* is a single copy gene that encodes the second largest subunit of the RNA polymerase II, the enzyme that transcribes pre-mRNA (Liu et al. 1999; Matheny et al. 2007). The *RPB2* gene possesses 12 highly conserved domains across kingdoms that have been used to design PCR primers (Liu et al. 1999). The *RPB2* gene was used in combination with other genomic regions to infer phylogenetic relationships at the species level for the genera *Cortinarius* and *Inocybe* (Froslev et al. 2005; Matheny 2005). In the present study, the authors explored use of the *EF1α* and *RPB2* genes to infer phylogenetic relationships among members of the *P. eryngii* species complex.

Materials and methods

Fungal cultures

A total of 39 isolates of *Pleurotus* spp. were used, representing six species (*Pleurotus dryinus* (Pers.: Fr.) Kumm., *Pleurotus ostreatus* (Jacq.: Fr.) Kumm., "*P. sapidus*" (Schulz.) Sacc. [name entered in the Pennsylvania State University Mushroom Culture Collection, PSUMCC], *Pleurotus tuberregium* (Fr.) Sing., *Pleurotus cornucopiae* (Paul.: Pers.) Roll. and *Pleurotus cystidiosus* Miller) and four varieties (*eryngii*, *ferulae*, *elaeoselini* and *nebrodensis*) of the *P. eryngii* species complex (Table 1). Isolates with codes "WC" were obtained from the PSUMCC (Table 1). Cultures in that collection are permanently stored in liquid nitrogen (−196 °C and 10 % glycerol).

Culture conditions and extraction of DNA

Isolates of *Pleurotus* spp. were grown for 15–20 d in 30 ml sterile potato dextrose broth (PDB) contained in 125 ml Erlenmeyer flasks. Broth and mycelium were filtered through miracloth® (Calbiochem). The mycelium was rinsed with sterile-distilled water and squeezed twice, placed in 2 ml eppendorf tubes, lyophilized, and stored in a glass desiccator at room temperature. Lyophilized mycelium was manually ground with a micropestle in a 1.5 ml Eppendorf tube. DNA extractions were performed using a DNeasy® plant mini kit (Qiagen) according to manufacturer's instructions. DNA concentrations were visualized and estimated by gel electrophoresis and a Beckman DU®640B spectrophotometer, respectively. DNA stock solutions were adjusted to 20 ng/μl with sterile, distilled water and stored at −20 °C until used.

PCR amplification of partial EF1α and RPB2 genes

The set of primers EF116OR (5′ CCGATCTTGTAGACGTCCTG 3′) and EF595F (5′ CGTGACTTCATCAAGAACATG 3′) was used to amplify a portion of the *EF1α* gene targeting exons 4–6 and expanding introns 4 and 5 (Wendland & Kothe 1997; Marongiu et al. 2005). Two sets of primers were used to amplify regions of the *RPB2* gene. Primers fRPB2 5F (5′ GAYGAYMG WGATCAYTTYGG 3′) and bRPB2 7.1R (5′ CCCATRGCY TGYTTMCCCAT DGC 3′) amplify a fragment of ~1100 bp targeting exons 3–5 (domains 5–7). Primers b6.9F (5′ TGGAC NCAYTGY GARATYCAYCC 3′) and b11R1 (5′ TGGATYTTG TCRTC CACCAT 3′) amplify a fragment of ~1100 spanning exon 4–5 (domain 7–11) (Liu et al. 1999; Matheny 2006). The schematic structure of the *EF1α* and *RPB2* genes and the target sections for primer recognition are available as Supplementary material (Fig S1). The PCR master mix from Promega (*Taq* DNA polymerase) was used to carry out PCR reactions (25 μl) with primers (10 μM each) and DNA template (60 ng). PCR cycling was done in a PTC-100™ Programmable Thermal Controller (MJ Research). PCR cycle conditions for *EF1α* amplifications were 94 °C/4 min; 36 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/1 min; and a final extension step of 72 °C/10 min. PCR conditions for amplification of the *RPB2* gene were 94 °C/5 min; 35 cycles of 94 °C/1 min, 57 °C/1 min, 72 °C/90 s; and a final extension step of 72 °C/10 min. PCR products were visualized by electrophoresis in 1 % agarose gels stained with ethidium bromide (0.16 μl/ml). DNA loading buffer (5×; 2 μl) + 5 μl PCR product were loaded on the gel and electrophoresed for approximately 30 min at 2.3–3.3 V/min.

DNA sequencing

PCR products were purified using ExoSAP-IT® (USB) according to manufacturer's instructions. Purified DNA template was concentrated (SPD1010 SpeedVac®System) or diluted to 40 ng/μl for sequencing. The forward primer for *EF1α* (EF595F) and reverse primers for *RPB2* (b11R1 and bRPB2 7.1R) were used for sequencing (1 μM each). DNA template and primer were transferred to 96-well μltraAmp™ PCR plates